

KAMIYA BIOMEDICAL COMPANY

***In Vitro* Nitric Oxide (Nitrite/Nitrate) Assay Kit (Colorimetric)**

**For the quantitative measurement of NO in various
samples by $\text{NO}_2^-/\text{NO}_3^-$ determination**

Cat. No. KT-943

For Research Use Only. Not for use in diagnostic procedures.

Product Information

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INTRODUCTION

Reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) work together to damage cells and have been implicated in the pathogenesis of several disease states. RNI are a family of molecules derived from nitric oxide (NO) and superoxide anion (O_2^-), produced via nitric oxide synthase (NOS) and NADPH oxidase, respectively. Nitric oxide is an established mediator in vascular diseases, diabetes, renal ischemia, atherosclerosis, inflammatory diseases, and cancer. However, because of its extremely short half life, direct quantitation of NO production remains challenging and unsuitable for most detection systems. Therefore, the common method for measurement of total NO has become the sum of its final oxidized products, nitrite (NO_2^-) and nitrate (NO_3^-) (Figure 1).

Nitric Oxide (Nitrite/Nitrate) Assay Kit is a simple, colorimetric assay that quantitatively measures NO in various samples by NO_2^-/NO_3^- determination. First, the nitrate (NO_3^-) in the sample is converted to nitrite (NO_2^-) by nitrate reductase enzyme (Figure 2). Next, total nitrite is detected with Griess Reagents as a colored azo dye product (absorbance 540 nm). Each kit provides sufficient reagents to perform up to 100 assays using a 96-well microtiter plate format, including blanks, calibrators and unknown samples. The kit is suitable for serum, plasma, urine, saliva, lysates, and media (see Preparation of Samples) with detection sensitivity limit of $\sim 2 \mu M$ (in 50 μL sample volume).

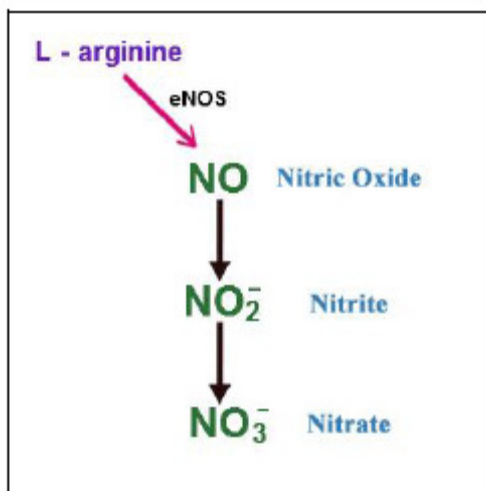


Figure 1: Oxidation of L-arginine to nitrate.

ASSAY PRINCIPLE

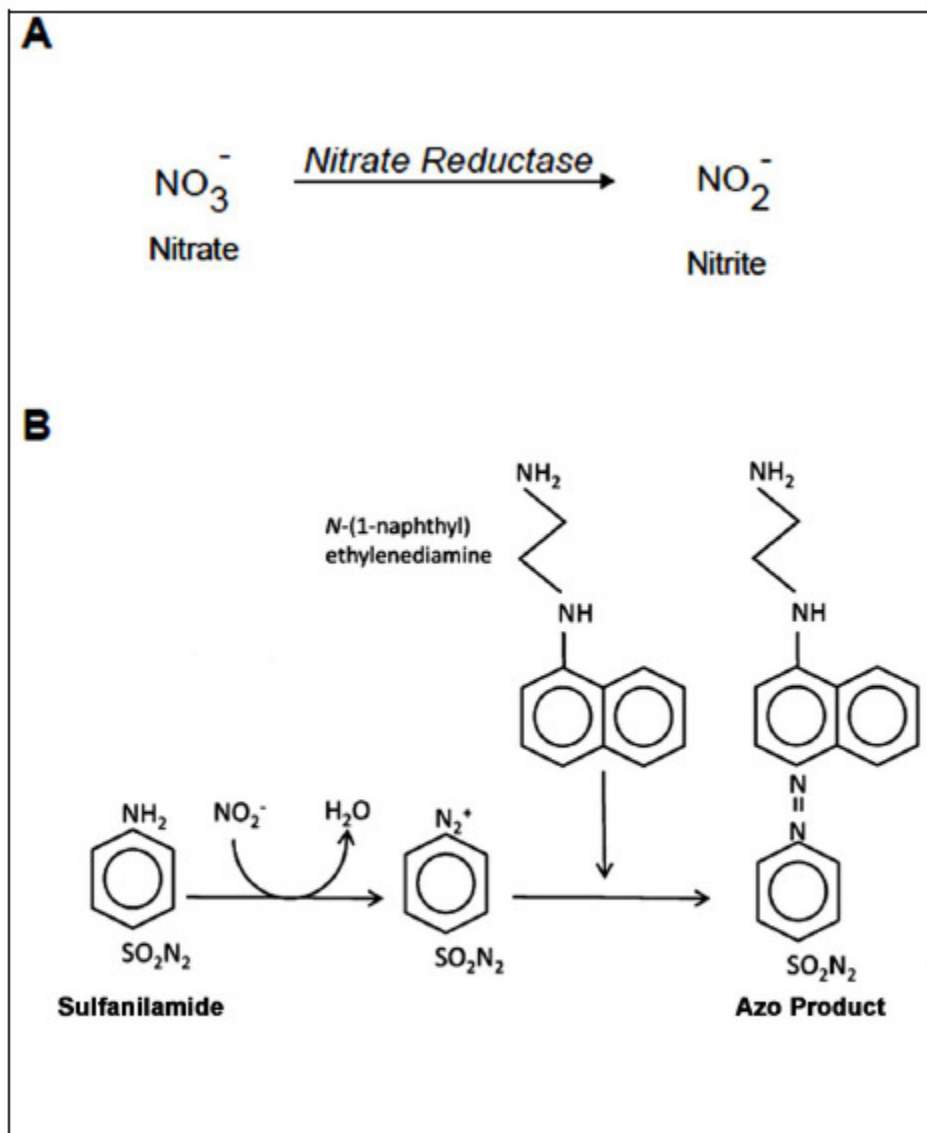


Figure 2: Conversion of nitrate to nitrite by nitrate reductase (A), followed by the Griess Reaction (B).

COMPONENTS

1. Nitrite Calibrator: One 500 μL vial of 14 mM sodium nitrite.
2. Nitrate Calibrator: One 500 μL vial of 14 mM sodium nitrate.
3. Enzyme Mixture: Three 1.4 mL vials.
4. Enzyme Cofactor: One 20 μL amber vial.
5. Griess Reagent A: Three 1.8 mL vials.
6. Griess Reagent B: Three 1.8 mL vials.

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Deionized or distilled water
2. PBS for sample dilution
3. 10 kDa MWCO ultrafilter (for high protein content samples)
4. 96-well microtiter plate
5. Microtiter plate shaker
6. 10 μL to 1000 μL adjustable single channel micropipettes with disposable tips

7. 50 μ L to 300 μ L adjustable multichannel micropipette with disposable tips
8. Multichannel micropipette reservoir
9. Microplate reader capable of reading at 540 nm

STORAGE

Store the entire kit at -80°C . Avoid multiple freeze/thaws by aliquoting. The Enzyme Cofactor is light sensitive and should be maintained in amber tubes.

REAGENT PREPARATION

- Nitrite and Nitrate Calibrators should be thawed/maintained at 4°C during assay preparation. Both are stable for 2 weeks at 4°C . For longer term storage, each should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.
- Enzyme Mixture should be thawed/maintained at 4°C during assay preparation. The solution is stable for 1 week at 4°C . Any unused material should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

Note: This component is provided in multiple tubes to minimize multiple freeze/thaws.

- Enzyme Cofactor should be thawed/maintained at 4°C during assay preparation. The stock solution is stable for 1 week at 4°C . Immediately before use, dilute the Enzyme Cofactor 1:100 with deionized water. Stir to homogeneity. Do not store diluted solutions. Any unused stock material should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

- Griess Reagent A and Reagent B should be thawed/maintained at 4°C during assay preparation. Both are stable for 4 weeks at 4°C . For longer term storage, each should be aliquoted and frozen at -80°C to avoid multiple freeze/thaws.

Note: This component is provided in multiple tubes to minimize multiple freeze/thaws.

Preparation of Nitrite or Nitrate Calibrators

- Thaw the nitrite or nitrate calibrators at 4°C and mix well. Freshly prepare a dilution series of calibrator in the concentration range of 0 μM – 140 μM by diluting the calibrator stock solution (provided at 14 mM) in deionized water (Table 1) or desired sample buffer.

Calibrator Tubes	14 mM Nitrite or Nitrate Calibrator (μL)	DI Water or Desired Buffer (μL)	Nitrite or Nitrate Calibrator (μM)
1	10	990	140
2	500 of Tube #1	500	70
3	500 of Tube #2	500	35
4	500 of Tube #3	500	17.5
5	500 of Tube #4	500	8.75
6	500 of Tube #5	500	4.38
7	500 of Tube #6	500	2.19
8	0	500	0

Table 1. Preparation of Nitrite or Nitrate Calibrators

Preparation of Samples

- Plasma: Collect plasma with an anticoagulant such as heparin, citrate or EDTA and mix by inversion. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C .

Note: Citrate or EDTA plasma is recommended. Heparin is known to interfere with the assay when samples contain $> 1 \text{ IU/mL}$; however, ultrafiltering will remove average MW heparin (15 kDa and larger). If low MW heparin is used, a 3 kDa MWCO ultrafilter should be used.

- Serum: Collect serum with no anticoagulant. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C .

- Urine: Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Urine must be diluted before assaying (typically 1:10 or greater in PBS). Use immediately or store at -80°C.
- Saliva: Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.
- Culture Media: Media known to contain high levels of nitrite/nitrate (e.g. RPMI) should be avoided. Remove particulates/debris in samples by centrifugation or filtration. To account for any background caused by media components, nitrite/nitrate calibrators should be prepared in the same media. Phenol red or FBS raise background values slightly.
- Cell and Tissue Lysates: Remove particulates/debris in samples by centrifugation or filtration. Samples must then be filtered through a 10 kDa MWCO ultrafilter before assaying (to reduce protein interference and turbidity). Use immediately or store at -80°C.

Potential Interference from Sample Components

Components known to interfere with the Griess Reaction are antioxidants, nucleophiles, and compounds containing sulfur (e.g. azide, ascorbic acid, cysteine, glutathione, DTT, β -ME) at concentrations of 10 μ M and above. Samples containing high concentrations of SDS (~ 0.1 %) are also incompatible. However, to accurately determine the degree of total interference, both the nitrate and nitrite calibration curves should be prepared in water vs. sample buffer. Dilution of the buffer, and ultimately samples, may be required to completely eliminate interference. Even with some interference, accurate quantitation can be achieved by running calibrators in the same buffer as samples, although kit sensitivity may be compromised.

ASSAY PROCEDURE

Note: This kit may be used for measurement of nitrite only, or for total nitrite plus nitrate through nitrate reduction. If desired, nitrate only levels in samples may be calculated by subtracting the nitrite only level from the total nitrite plus nitrate.

I. Measurement of Nitrite only

Note: Each nitrite calibrator and sample should be assayed in duplicate or triplicate. A freshly prepared calibration curve should be used each time the assay is performed.

1. Add 50 μ L of the nitrite calibrators, samples, or blanks to the 96-well microtiter plate.
2. Add 50 μ L of PBS to each well, adjusting the total volume to 100 μ L per well.
3. Add 50 μ L of Griess Reagent A to each well.
4. Immediately add 50 μ L of Griess Reagent B to each well.
5. Incubate the plate at room temperature for 10 minutes, allowing color development.
6. Read absorbance at 540 nm on a microplate reader.
7. Calculate the concentration of nitrite within samples by comparing the sample absorbance to the calibration curve. Negative controls (without nitrite) should be subtracted.

II. Measurement of Total Nitrite/Nitrate via Nitrate Reduction

Note: Each nitrate calibrator and sample should be assayed in duplicate or triplicate. A freshly prepared calibration curve should be used each time the assay is performed.

1. Add 50 μ L of the nitrate calibrators, samples, or blanks to the 96-well microtiter plate.
2. Maintain Enzyme Mixture and Cofactor at 4°C. According to Table 2 (below), prepare the desired volume of Enzyme Reaction Mixture (based on the # of tests) in the following sequence:
 - a. In a tube, add the appropriate volume of Enzyme Mixture.
 - b. Next, add the corresponding volume of diluted, Enzyme Cofactor Solution (see Preparation of Reagents).

Note: Enzyme Cofactor is diluted 1:100 in deionized water immediately before use.

- c. Mix well and immediately use.

# of Tests in 96-well Plate (50 μ L/test)	Enzyme Mixture (mL)	Diluted Cofactor Solution (mL)	Total Volume of Enzyme Reaction Mixture (mL)
100	4	1	5
50	2	0.5	2.5
25	1	0.25	1.25

Table 2. Preparation of Enzyme Reaction Mixture

3. Transfer 50 μ L of the above Enzyme Reaction Mixture to each well (already containing 50 μ L of nitrate calibrator or sample).
4. Cover the plate wells to protect the reaction from light.
5. Incubate at room temperature for 1 hour on an orbital shaker.
6. Add 50 μ L of Griess Reagent A to each well.
7. Immediately add 50 μ L of Griess Reagent B to each well.
8. Incubate the plate at room temperature for 10 minutes, allowing color development.
9. Read absorbance at 540 nm on a microplate reader.
10. Calculate the concentration of nitrate within samples by comparing the sample absorbance to the calibration curve. Negative controls (without nitrate) should be subtracted.

EXAMPLE OF RESULTS

The following figures demonstrate typical Nitric Oxide Assay Kit results. One should use the data below for reference only. This data should not be used to interpret actual results.

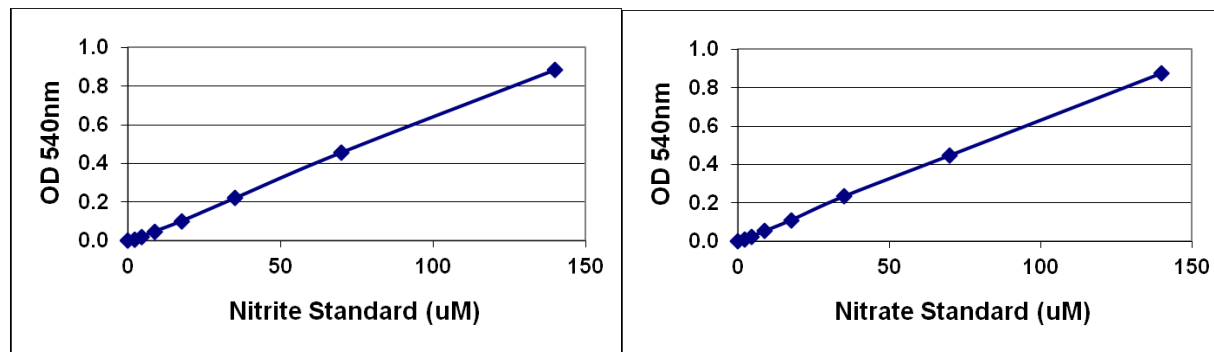


Figure 3: Nitrite and Nitrate Calibration Curves. Nitrite (left) and nitrate (right) calibration curves were performed according to the Assay Protocol.

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